Nucleic acid detection method

The present invention relates to nucleic acid
detection methods, in particular to quantitative nucleic
acid detection methods.

Detection, especially quantitative detection of a
particular nucleic acid sequence, as an indication of
the presence of an organism e.g. a pathogen in a
clinical sample or a contaminant in a food or
environmental sample, such as toxin-producing
cyanobacteria in water sources, or of mRNA to show a
change in transcription levels, is a valuable
microbiological tool. In addition, in the diagnostic or
forensic use of nucleic acid analysis or in the study of
polymorphisms, full sequencing of the target nucleic
acid may be unnecessary where the detection of a single
base variation or mismatch is sufficient to give a
positive identification. Such a single base variation
or mismatch may, for example, arise from allelic
variation or polymorphism, a point mutation, or any
deletion or insertion of genetic material where the
detection of a single abnormal or species specific base
will give the required information.

Through our work in the development of methods for
the detection of bacteria in water, we have developed a
new nucleic acid detection method suitable for a wide
variety of applications in the environmental,
agricultural, food, veterinary, health and medical
fields and indeed as a general tool in molecular
biology.

There are a number of techniques available for the
analysis of nucleic acids including the manufacture of
synthetic oligonucleotide probes, particularly labelled
probes, for hybridisation to target sequences; *in vitro*
amplification of target nucleic acid sequences by PCR
and other related amplification methods and (automated)

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direct DNA sequencing. These have led to the development of novel approaches for the detection and characterisation of nucleic acids in environmental monitoring (Bej, A.K. and Mahbubani, M.H. [1994] in PCR Technology: Current Innovations p 327-339 and Bowman, J.P. and Sayler, G.S. [1996] in Molecular approaches to environmental microbiology p 63-97). There are three main strategies for quantification of amplified DNA; i.e. size separation by electrophoresis, hybridization to capture probes, and real-time detection. The problems with the gel electrophoresis method are the detection of multiple targets in a single reaction, and interpretation of the results. Size separation detection of multiplex amplifications is also difficult to achieve because the amplification ratios of amplicons with different sizes are dependent on DNA quality.

The capture probe assay is based on hybridization of the entire amplified fragments. Evidently, this assay is not suitable for separation and quantification of homologous amplicons, e.g. products of competitive amplifications. The different amplicons will form sandwich hybridizations at the homologous sites, leading to the capture of both target and non-target fragments, even if the capture site is discriminating.

The ABI PRISM™ 7700 Sequence Detection System (Perkin Elmer, Foster City, California) provides real-time quantitative PCR amplification. However, multiplex assays are limited by the number of fluorochromes available and their overlapping fluorescent spectra.

It is always an aim in nucleic acid detection methods to increase their specificity, ie. to reduce non-specific binding or detection of a non-specific background signal. A separate but associated goal is to increase the sensitivity of the detection method, ie. to allow measurement of very small amounts of target nucleic acid.

It would also represent a significant advantage if

the method allowed the detection and quantification of several, preferably a large number such as 10 or more, polymorphic sites in a single reaction e.g. allowing the detection and quantification of several different target organisms in a single multiplex assay.

We have developed a convenient detection method which provides good sensitivity and specificity and the ability to detect and quantitate a large number of polymorphic sites in a single reaction. In particular, the methods represent significant advantages over those employing agarose gel electrophoresis or direct detection of the amplified DNA in the quantification.

The invention thus provides a method of detecting a target nucleotide sequence in a nucleic acid molecule, which comprises:

(a)   binding of an oligonucleotide probe to said nucleic acid molecule;

(b)   selective labelling of the bound oligonucleotide probe in the presence of said target nucleotide sequence;

(c)   hybridisation of the labelled oligonucleotide to a complementary sequence; and

(d)   subsequent detection of the label.

The method of the invention can be used in the detection of all target nucleotide sequences, such as DNA sequences, particularly DNA resulting from PCR amplification cycles. The DNA may be native or cDNA formed from mRNA by reverse transcriptase. The DNA may be single or double stranded, linear or circular. The target nucleic acid may be RNA, e.g. mRNA or in particular ribosomal RNA which is present in a cell in multiple copies, for example 3,000-20,000 copies per cell.

In the context of the present invention, the term "nucleotide sequence" may refer to a 'sequence' of only one nucleotide in length, where the nucleic acid of interest differs by only one nucleotide from other (non-

target) sequences which it is not desired to detect, such as in the case of detection of a point mutation or polymorphism. More usually, the nucleotide sequence to be detected is characteristic of a particular nucleic acid or group of nucleic acids or of a particular organism or group of organisms e.g. a species, where it is desired to detect the presence of that target (e.g. the nucleic acid/or organism) in a sample containing a number of different molecules or organisms, such as in the detection of particular bacteria e.g. in a clinical or environmental sample.

The oligonucleotide probe may comprise 5 to 50, preferably 10 to 40, more preferably 20 to 30 nucleotides. The probe is selected to bind to the target nucleic acid, namely the nucleic acid molecule containing the target nucleotide sequence. The oligonucleotide is conveniently sufficiently large to provide appropriate hybridisation to the target nucleic acid, yet still reasonably short in order to avoid unnecessary chemical synthesis. Methods for oligonucleotide production are standard in the art.

As discussed above, the target nucleic acid may be DNA, cDNA or RNA. The oligonucleotide probe is designed to bind to a target region in the nucleic acid molecule containing the target nucleotide sequence. "Target region" is used herein to refer to the sequence of nucleic acid which binds to the oligonucleotide probe. This target region may be in the form of a "signature sequence" which characterises a particular nucleic acid or group of nucleic acids or organisms etc. or may be the section of nucleic acid immediately preceding a single base position of interest, if a polymorphism, point mutation, insertion or deletion is to be detected.

It is of course desirable that the target region be absent from any non-target molecules in the sample in order to introduce selectivity between target and non-target nucleotide sequences. This specificity of

binding may be achieved by incorporating into the probe
regions of complementarity or substantial
complementarity to the desired target region and/or
mismatches to non-target sequences. The region of
5   complementarity may be present at terminal or internal
segments of the probe, or both.

Conventionally, nucleic acid detection methods have
involved labelling of the oligonucleotide probe prior to
hybridisation to the target nucleic acid sequence.
10   However, in our method, labelling of the probe is
dependent upon the sequence of the nucleic acid molecule
to which the probe is bound. In other words labelling
of the probe is template-sequence dependent (i.e.
sequence-specific) - incorporation of the label will
15   only occur in the presence of a desired or selected
"sequence" in the target molecule, which may be in the
target region or in the target nucleotide sequence
itself, or it will occur selectively ie. the step of
label incorporation into the oligonucleotide probe may
20   be discriminating (ie. based on a "mini-sequencing"
principle). This adds an extra dimension of selectivity
(specificity) to the process; oligonucleotide probe
binding will initially discriminate against non-target
nucleic acid but some binding to non-target sequences
25   may be expected. If the probe itself were labelled
prior to binding, then further discrimination would not
be possible.

The present invention, on the other hand, adds a
step of selectively (sequence-specifically)
30   incorporating a label, in the presence of the target
nucleotide sequence, into the oligonucleotide probe.
This selectivity may be achieved, for example, by
incorporation of the label only when the target
nucleotide sequence is present, or by incorporating the
35   label in a selective or discriminating manner such that
the target nucleotide sequence may be discriminated, or
both.

Conveniently, selective labelling may be achieved using labelled nucleotides ie. by incorporating (or not incorporating) into the oligonucleotide probe a nucleotide carrying a label. In other words selective labelling may occur by chain extension of the oligonucleotide probe using a polymerase enzyme which incorporates a labelled nucleotide. In the case of an RNA target, the polymerase will be a reverse transcriptase enzyme. Conditions for chain extension (ie. base incorporation) and suitable polymerase enzymes are well known in the art and widely described in the literature.

Selective labelling of the oligonucleotide is preferably achieved by incorporation of a labelled dideoxynucleotide.

The term dideoxynucleotide as used herein includes all 2'-deoxynucleotides in which the 3'-hydroxyl group is absent or modified and thus, while able to be added to the oligonucleotide probe in the presence of a polymerase, is unable to enter into a subsequent polymerisation reaction. This means that only one base can be added to the probe.

Chain termination in this manner allows the reaction to be controlled, and the assay to be quantitated, if desired (see later).

Advantageously, as described above, the present invention involves chain extension of the oligonucleotide probe to incorporate a label. The present invention permits selective labelling to be achieved both by incorporation of a labelled nucleotide in a selective manner, (ie. discriminating on the basis of the label, either its presence or absence, or by selective detection of a particular label) or by nucleotide incorporation in a selective manner such that incorporation of a nucleotide (whether labelled or not) occurs only in the presence of the target nucleotide sequence (or more accurately, that the efficiency of

incorporation is much reduced where the target
nucleotide sequence is not present) (ie. discriminating
on the basis of nucleotide incorporation or non-
incorporation), or both.

As an example of such a method, if the target
sequence is a key differentiating base, e.g. if the base
in the nucleic acid molecule immediately adjacent to the
target region to which the probe binds, is a cytosine
residue, then labelled dideoxyguanine triphosphate
(ddGTP) would be added to the reaction mixture for
incorporation. If the probe had bound to nucleic acid
at a non-target sequence, then it is unlikely that the
next nucleotide is the same as the key base, and the
labelled ddGTP is then unlikely to be incorporated in
the oligonucleotide ie. incorporation of the
oligonucleotide takes place only (or largely or
substantially only) in the presence of target. The
above method of differentiation applies *mutatis mutandis*
if the key base is guanine, adenine, thymine or uracil.

In order further to reduce the likelihood of
misincorporation of the labelled dideoxynucleotide,
unlabelled dideoxynucleotide triphosphates may also be
added to the reaction mixture. Using the above example,
where labelled ddGTP is added, unlabelled ddCTP, ddATP
and ddTTP would be added (where the target nucleic acid
molecule is DNA). If only one ddNTP is present, then
despite the rules of base pairing, there may be a risk
that it will be incorporated where the key base is not
its natural base pair, albeit at a low frequency. This
may cause a background or noise signal in the assay.
The inclusion of the other ddNTPs means that these will
be incorporated preferentially, according to the normal
rules of base pairing (C-G and A-T/U), and
misincorporation of the labelled ddNTP is significantly
reduced. The use of unlabelled ddNTPs thus comprises a
preferred embodiment of the present invention.

In a further preferred embodiment of the present

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invention, selectivity of the labelling reaction is
further increased by incorporation of mismatches
immediately upstream of the key base. Thus, the
oligonucleotide probe is designed with a mismatch or
5   mismatches at the 3'-end to non-target nucleotide
sequences, and the presence of the mismatch(es) will
reduce the likelihood of oligonucleotide extension.
Therefore, even if the next base after the end of the
bound oligonucleotide is by chance the same as the key
10   base, in non-target sequences incorporation of the
labelled ddNTP will be reduced where there is preceding
mismatch between the probe and the nucleic acid to which
it has bound.

A large number of suitable labels and labelling
15   methods are known in the art and described in the
literature. Any detectable or signal-generating label
or reporter molecule may be used. Convenient labels
include colorimetric, chemiluminescent, chromogenic,
radioactive and fluorescent labels, but enzymic or
20   antibody-based labelling methods or signal-generating
systems may also be used. Thus the term "label" as used
herein includes not only directly detectable signal-
giving moieties, but also any moiety which generates a
signal or takes part in a signal generating reaction.
25   Fluorescein or other fluorescently labelled ddNTPs are
particularly suitable, which allow detection directly by
fluorescence or indirectly by antibody interactions.
These are commercially available from e.g. NEN/Dupont.
ddNTPs can be labelled by e.g. $[^{35}S]$, $[^{3}H]$ or $^{32}P]$ as
30   described in Syvänen, A.C. et al. Genomics 8, [1990],
684-692.

In order to enhance sensitivity, more than one
labelling step may be performed. In other words, the
selective labelling step may be repeated one or more
35   times, ie. performed cyclically. Increasing the number
of cycles increases the sensitivity, and also when the
assay is performed quantitatively (see below), the

quantitative range of the method, by improving the label
saturation of the probes. In order to facilitate cyclic
labelling, a thermostable polymerase enzyme may be used
in the label-incorporation step. Conveniently, the

5   number of cycles of the selective labelling step may be
from 1 to 50, e.g. 10 to 30 but this may be varied
according to choice. An appropriate or convenient
number of cycles for a given system may be determined by
routine experiments.

10   Minisequencing methods to detect multiple single-
nucleotide polymorphisms are known which involve single-
nucleotide extension reactions with fluorescently
labelled dideoxynucleotides (Pastinen, T. et al.
Clinical Chemistry (1996) Vol. 42, 9, 1391-1397). In

15   this case, the minisequencing reaction products were
analysed by gel electrophoresis. In contrast, the
present method involves a hybridisation reaction between
the labelled oligonucleotide and its complementary
nucleic acid sequence prior to detection and analysis.

20   Thus, following the selective labelling step(s),
the labelled oligonucleotide probe is hybridised to a
complementary nucleotide sequence. Generally, this
involves separation of the labelled oligonucleotide
probe from the previous reaction mixture. Label

25   incorporation by incorporation of a labelled nucleotide,
results in the so-labelled oligonucleotide probe
hybridised to the target nucleic acid molecule. Strand
separation to separate the probe may be achieved using
denaturation methods conventional in the art e.g. high

30   temperature heating, treatment with high pH (alkali)
etc. Step (c), hybridisation to a sequence
complementary to the probe, may then take place. This
"complementary sequence" according to the invention may
be any nucleotide sequence containing or comprising a

35   region of absolute or substantial complementarity to the
probe such that binding of the probe to the
"complementary sequence" can occur. The complementary

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sequence may be DNA or a modification thereof such as
PNA. Conveniently, the complementary sequence may take
the form of the complement of the probe (ie. a sequence
fully complementary to the probe). It will be
understood however, that this is not absolutely
essential and that the "complement" may be contained in
a longer sequence, may be partial or truncated, and/or
may contain sequence variation (ie. less than perfect
complementarity) as long as binding of the probe to the
"complementary sequence" may still occur.

In a preferred embodiment, the complementary
sequence is immobilised on a solid support. Suitable
immobilising supports to which the complements can be
attached are known in the art and include any of the
well known supports or matrices which are currently
widely used or proposed for immobilisation, separation
etc. These may take the form of particles, sheets,
gels, filters, membranes, fibres, capillaries, chips or
microtitre strips, tubes, plates or wells etc. Methods
of immobilising or attaching oligonucleotides to solid
supports are likewise known in the art. Particularly
preferred are membrane strips (commercially available
from NEN and known as GeneScreen) on to which the
complementary sequences may be spotted and then U.V.
cross-liked, or DNA chips (microchips, glass chips) now
common in molecular biology procedures. The use of such
solid supports, in particular chips which can carry an
oligonucleotide array ie. a number, e.g. up to 250, of
different oligonucleotides complementary to the
oligonucleotide probes used in the earlier part of the
assay, represents a particularly preferred embodiment of
the invention.

Hybridisation step (c) permits the quantitative
separation of the labelled probes. This is advantageous
in the context of a quantitative assay.

Following the hybridisation step, the label on the
oligonucleotide probe is detected. As mentioned above

in the context of the label, the detection may be by any
means known in the art, depending on the label selected.
Signal amplification may also be used, if desired, as
described in the art (see, for example, P. Komminoth and
M. Werner, Target and Signal Amplification, approaches
to increase the sensitivity of in situ hybridisation,
1997 Histochem. Cell Biol. 108:325-333). Detection may
also be qualitative, or quantitative or semi-
quantitative. Thus, for example, a visual e.g.
colorimetric, chromogenic, fluorescent or other signal
detectable from the label may be used to provide a
simple yes or no indication of the presence or absence
of the target nucleotide sequence. Advantageously,
however, the detection is such as to yield a
quantitative assessment of the amount of target
nucleotide present in the sample. This may be an
absolute indication of the amount of target present or
some other quantitative or semi-quantitative indicator
e.g. a percentage, ratio, or similar indicator, e.g.
relative to total nucleic acid in the sample.

Quantitation of the target nucleotide sequence may
further be correlated to a quantitation of the number of
organisms or cells containing the target in question.
Thus, in further aspects, the invention provides methods
of determining the amount of a target nucleotide
sequence or the number of cells containing a target
nucleotide sequence using the nucleic acid detection
assay hereinbefore described.

As mentioned above, the target nucleic acid may be
any DNA or RNA molecule. It may thus be directly the
nucleic acid of a target cell or organism. It may be
desirable, however, to amplify the nucleic acid prior to
detection using the assay method of the invention and
advantageously the target nucleic acid may thus be an
amplicon of an in vitro amplification reaction.
Amplification may however also take place by other means
including in vivo cloning methods. Any method of in

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vitro amplification may be used, including both linear
and exponential methods. Representative methods thus
include PCR, NASBA and ligase chain reaction. Due to
its convenience, PCR and its variants will commonly be
5   the method of choice.

In the context of a quantitative assay, a
quantitative *in vitro* amplification method may be
combined with the detection assay of the invention. In
particular, in a preferred embodiment of the methods of
10   the present invention, the oligonucleotide probe binding
step is preceded by a competitive PCR reaction. A
variety of methods of competitive PCR exist and are
described in the literature. Any of the known
techniques may be used. (For a review see e.g. Srebert,
15   P.D. and Larrick, J.W. [1992] Nature, 369, 557-558).

In such a method, nucleic acid incorporating the
target nucleotide sequence is co-amplified with a
selected competitor molecule containing the same primer
template sequences as the target molecule and thus the
20   competitor competes with the target for PCR primer
binding and amplification. The competitor comprises a
means for differentiating from the target, conveniently
a recognition sequence for binding to a competitor-
specific oligonucleotide probe, analogous to the
25   "target-specific" oligonucleotide probe described in the
context of the detection assay above (ie. the probe of
step (a) above). The recognition sequence is thus
analogous to the "target region" of the oligonucleotide
probe above. A single known amount of competitor may be
30   added, for comparison to a standard curve, or a series
of amplifications may be performed using varying amounts
of competitor e.g. a dilution series. Primers
complementary to sequences flanking the target
nucleotide sequence in the nucleic acid molecule of
35   interest (and to the same sequences in the competitor)
are added and PCR amplification is performed.
Competitive PCR enables the detection to be performed

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with a built in reference to provide more accurate
analysis of the results. A standard curve may be
constructed using standard techniques conventional in
the art.

5   In such a competitive PCR step, amplicons are
produced deriving from both target and competitor DNA.
The competitor amplicon is subjected to a detection
assay procedure in an analogous manner to the target
amplicon, as described above. The amplified competitor
10   sequence includes a known recognition sequence which
behaves in the same way as the target region in the
target nucleic acid molecule under detection. For
example, an oligonucleotide probe binding selectively to
this recognition region of the competitor is added to
15   the reaction mixture at the same time as the probe to
the target region of the target molecule is added, both
oligonucleotides being designed to be single base
extended with a labelled ddNTP when bound to their
respective target sequences. A complementary sequence
20   to the competitor oligonucleotide probe which has been
labelled following binding to the competitor is also
provided, preferably immobilised, and the labelled
oligonucleotide probe hybridises to this complementary
sequence before detection. The labels of the target and
25   competitor oligonucleotides can then be detected and, in
particular, the relative strengths of the measured
signal used to evaluate the amount of target nucleic
acid which was present in the original sample given the
known amount of competitor added to the system. This
30   aspect of the invention is represented in Figure 1. It
is not necessary that the label for the competitor and
the target sequence are different, indeed they are
preferably the same. Differentiation between competitor
and target, as between a plurality of different target
35   sequences, is preferably achieved spatially; the
position of the complementary sequence to each
oligonucleotide probe on e.g. the microchip array is

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known and the strength of signal at each position is
measured.

Titration experiments can be used to determine the
optimum amount of competitor to be used in any
particular assay, as described in the following
Examples. The amount of competitor selected preferably
allows detection of a low frequency of target nucleic
acid molecules and reproducible amplifications.
Suitable concentrations may vary from 6×10^{-8} to 6×10^{-10}
pmol, preferably from 4×10^{-9} to 8×10^{-9} pmol.

Preferably, sufficient cycles of PCR are performed
so that a saturation level is achieved; in that way the
amplified DNA contains substantially the same ratio of
target DNA to competitor DNA as that present before PCR.

It is advantageous to provide conditions such that
the competitor and target DNA are amplified equally
efficiently by PCR and it is therefore desirable that
the size and GC-content of each fragment is kept
substantially the same. In some instances it will also
be necessary to take into account the efficiency of the
cell lysis of the sample which can make some of the
genomic DNA less available for the PCR. In addition,
the target DNA may be part of chromosomal DNA in the
initial cycles, while the competitor DNA may be, for
example, in the form of a small plasmid. Thus it might
be necessary to adjust for the relative amplification
efficiencies of these early PCR cycles.

Alternative amplification strategies may also be
used in this aspect of the invention, for example NASBA
and ligase chain reaction.

Any suitable polymerase may be used, although it is
preferred to use a thermophilic enzyme such as Taq
polymerase to permit the repeated temperature cycling
without having to add further polymerase, e.g. Klenow
fragment, in each cycle.

As mentioned above, the target nucleic acid may be
cDNA synthesised from mRNA in the sample, and the method

of the invention is thus applicable to diagnosis on the
basis of characteristic mRNA. Such preliminary
synthesis can be carried out by a preliminary treatment
with a reverse transcriptase, conveniently in the same
system of buffers and bases to be used in the subsequent
amplification (e.g. PCR) steps. Since the PCR procedure
requires heating to effect strand separation, the
reverse transcriptase will be inactivated in the first
PCR cycle. When mRNA is the target nucleic acid, it may
be advantageous to submit the initial sample, to
treatment with an immobilized polydT oligonucleotide in
order to retrieve all mRNA via the terminal polyA
sequences thereof. Alternatively, a specific
oligonucleotide sequence may be used to retrieve the RNA
via a specific RNA sequence. The oligonucleotide can
then serve as a primer for cDNA synthesis, as described
in International Patent Application WO-A-90/11446.

The PCR (or other *in vitro*) amplification of target
and competitor nucleic acid provides a first level of
specificity but a low level of non-target/non-competitor
molecules may be amplified in this reaction, hence the
selective labelling of the oligonucleotide probes based
on signature sequences in the target and the competitor
amplicons according to the present invention. The
quantification is based on the signal ratio of target to
competitor alone and is not affected by any non-
target/non-competitor molecules which may have been
amplified in the first reaction.

When the main detection assay follows a PCR or
other *in vitro* amplification step based on chain
extension, then it is necessary to separate the dNTPs
from the previous reaction e.g. in the PCR product or to
inactivate them to ensure that they could not be
involved in subsequent elongation of the
oligonucleotides bound to target/competitor nucleic acid
in place of the terminating ddNTPs. Physical separation
of the dNTPs from the PCR product is possible by e.g.

gel electrophoresis, precipitation, affinity
purification, chromatography or filtration. However it
is preferred to inactivate the dNTPs by removing the
reactive phosphate groups by a phosphatase so that the
nucleotides can no longer be used for significant chain
elongation.

Target nucleic acid for use in the method of the
invention may be obtained or prepared by any convenient
technique in the art. Many different methods for
isolating nucleic acids from cells and organisms are
described in the literature and any of these could be
used.

For example, methods for the extraction of nucleic
acid from environmental samples are described by Bowman,
J.P. and Sayler, G.S. in Molecular Approaches to
Environmental Microbiology (1996), pp 63-97. However, a
particularly preferred embodiment of the present
invention combines a detection method as described above
with the solid-phase method for combined cell and
nucleic acid isolation described by us in Rudi et al.,
Appl. Environ. Microbiol. (1998), 64(1), pp 34-77.

The method of isolating nucleic acid from a sample
of cells which is described in this paper comprises:

(a) binding cells in the sample to a solid support
to isolate the cells from the sample;

(b) lysing the isolated cells; and

(c) binding nucleic acid released from said lysed
cells to said same solid support.

The bound nucleic acid may be used directly in a
detection assay of the invention or it may be released
from the support.

The detection assay of the present invention may
advantageously find application in any procedure where
it is desired to detect nucleic acids, for example in
any procedure relying on DNA or RNA identification, e.g.
diagnosis, detection of infections or contaminating
organisms or pathogens, clinical monitoring, forensics,

food and environmental safety and monitoring, or for
distinguishing between different cells or cell types,
mutation detection, analysis of polymorphisms, e.g. in
tissue typing etc. and as a general molecular biology

5   tool. The complete nucleic acid-based assay comprising
sample preparation, DNA amplification, selective
labelling and detection is of particular benefit in
quantifying the amount of toxin-producing cyanobacteria
in water sources. This combined isolation and detection

10   method comprises a particularly preferred embodiment of
the present invention. The assay provides a lower
detection limit of 200, preferably 100 cells/ml (of a
particular cyanobacteria against a background of 10^5
cells/ml of other cyanobacteria) and a quantitative

15   range of more than three orders of magnitude. The
methods described in principle above and in more detail
below are suited for automation, providing the means for
the development of high throughput systems for routine
environmental monitoring.

20   For example, water-blooms formed by cyanobacteria
have a relatively high frequency of toxicity (between 25
and 70%), and constitute a potential health hazard to
livestock and humans. This is particularly the case for
blooms of cyanobacteria belonging to the genus

25   *Microcystis*. Species of this genus can produce several
toxins, with the hepatotoxic microsystins being the most
potent. It would therefore be desirable if a method
could be developed which provided a reliable, quick and
convenient way to detect and quantify these bacteria.

30   The methods of the invention are particularly
suitable in this respect, in that by selection of
oligonucleotides to signature sequences within the
genome of different bacteria of interest, a single
sample can be analysed in one procedure for several

35   different microorganisms. Signature sequences for
distinct bacterial groups are widely known and published
in the literature, for cyanobacteria a classification

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system based on 16S rDNA sequence information can be
used (see e.g. J.R. Marchesi, T. Sato, A.J. Weightman,
T.A. Martin, J.C. Fry, S.J. Hiom, and W.G. Wade, 1998.
Design and evaluation of useful bacterium-specific PCR
5   primers that amplify genes coding for bacterial 16S
rRNA. *Appl. Environ. Microbiol.* 64:795-799; Turner, S.
(1998) Origins of algae and their plastids, pp 13-53 Ed.
Bhattacharya, D. NY: Springer-Verlag and Giovanni, S.J.,
Turner, S., Olsen, G.J., Barns, S., Lane, D.J. and Pace,
10   N.R. (1988) J. Bacteriol **170** pp 3584-92).

The Examples which follow describe a fully
integrated assay for analysis of complex cyanobacterial
communities. The methods of the invention are equally
suitable for use in the investigation of other microbial
15   communities.

Complementary sequences to the different labelled
oligonucleotides can be arranged on a single microchip
in a pre-determined manner to allow quantification of
the levels of different bacteria, preferably by
20   comparison of the ratio of competitor signal to the
signal for each bacterial group. Such multiplex
quantitative assays preferably use high density
oligonucleotide arrays immobilised on glass chips, with
subsequent direct detection of the fluorescein-label
25   (see e.g. Schena, M. et al., Science, (1996) 270: 467-
470; and R.J. Lipshutz, D.M. Morris, M. Chee, E.
Hubbell, M.J. Kozal, N. Shah, N. Shen, R. Yang, and S.P.
A. Fodor. Using oligonucleotide probe arrays to access
genetic diversity, Bio Techniques 19:442-447).

30   The invention also comprises kits for carrying out
the methods of the invention. These will normally
include at least the following components:

(a)   an oligonucleotide probe capable of binding to
a target nucleic acid molecule containing the target
35   nucleotide sequence (ie. directed to a target region
within the nucleic acid molecule;

(b)   means for selective labelling of the

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oligonucleotide probe; and

(c)   nucleotide sequences complementary to the oligonucleotide probes, preferably immobilised on a solid support.

The means for selective labelling will conveniently comprise a polymerase enzyme and at least a labelled nucleotide, preferably a labelled ddNTP, optionally with corresponding unlabelled nucleotides also present.

The above described invention will now be illustrated by the following non-limiting Examples in the field of cyanobacteria detection with reference to the drawings in which:

Figure 1 is a schematic representation of the quantitative labelling assay. A known concentration of competitor DNA was added to the purified target and co-amplified with the same primer pair (A). Two oligonucleotides, one complementary to an internal segment of the competitor and one complementary to an internal segment of the target were sequence specifically extended by a fluorescein labelled dideoxy cytosine by thermo-cycling (B). The labelled primers were then hybridized to their immobilized complements (C). Finally, a chromogenic detection of the label was performed (D), and the relative signal intensities determined.

Figure 2 (A) - shows gel results from a competitive PCR on dilution series of DNA isolated from *Microcystis aeruginosa* NIVA-CYA 43,b

(B) - shows the results of the labelling assay,

(C) - is a graph showing the intensities of the target signals relative to the total signal intensities.

The assays were performed on dilution series of purified DNA. The amount of DNA is given as genomic copies (assuming a genomic copy weight of 5 fg). In panel A, 10 μl of the products from the competitive PCR

reaction were loaded in each lane on a 1.5% agarose-gel
(containing 30 μg/ml of ethidium bromide) and
electrophoresed using 1 x TBE at 100 volts for 1 hour.
The products were visualized by U.V.-transillumination.
The labelling assay shown in panel B was performed as
described in the Examples. In panel C, the signal
intensities (measured as the difference in the average
pixel value in a 8 bits grayscale, between the signal
and the background) for the target in panel A measured
relative to the total signal intensities of both the
target and the competitor (●). Respective values for
the labelling assay in panel B are also shown (▲). The
pictures were taken with a Cohu High Performance CCD
Camera, and printed on a digital color printer
(Mavigraph UP-D1500CNE. Sony, Japan).

Figure 3 - is a graph showing the percentage of the
signal intensities for the target spots relative to the
total signal intensities, with mean value for all the
experiments, and error bars for standard deviation (3
degrees of freedom). Complete assay with 6×10^{-9} pmol
competitor on dilution series of *Microcystis aeruginosa*
NIVA-CYA 43 in different aqueous environments. Cells
were diluted in sterile water, water containing *Anabaena
Lemmermannii* NIVA-CYA 83/1 (10^5 cells/ml), water
containing *Planktothrix agardhii* NIVA-CYA 29 (10^5
cells/ml) and in water from Lake Akersvatnet, Norway
(sampled May 30, 1996). The complete assay including
solid-phase cell concentration and DNA purification was
performed as described in the Examples.

Figure 4 - (A) - shows the results of the labelling
assay following solid-phase cell concentration and DNA
purification as described in the Examples. Complete
assay with 6×10^{-8} pmol competitor on dilution series of
Microcystis aeruginosa NIVA-CYA 43 in water from Lake
Akersvatnet, Norway (sampled May 30, 1996).

(B) - is a graph showing the percentage
of the signal intensities for the target spots relative

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to the total signal intensities. The pictures were
taken with a Cohu High Performance CCD Camera, and
printed on a digital color printer (Mavigraph UP-
D1500CNE).

5   Figure 5 - (A) - shows membrane strips with probes.
                  (B) - shows signal intensities for the
respective probes.
                  (C) - shows phylogenetic position for
the different probes.

10   The probes were spotted on the membrane in the
following order: first row - pKO, pAP and pMI3; second
row - pMI2, pDK and pPL2; third row - pPL1, pAL and
pNOS; fourth row - pUN. The strains tested are for
membranes a1; *Aphanizomenon gracile* NIVA-CYA 103, a2;

15 *Anabaena lemermannii* NIVA-CYA 266/1, a3; *Nostoc* sp. NIV-
CYA 123, b1; *Phormidium* sp. NIVA-CYA 203, b2;
Planktothrix prolifica NICA-CYA 320, b3; *Microsystis
aeruginosa* NIVA-CYA 143, c1; *Microcystits flos-aquae*
NIVA-CYA 144, c2; *Pseudanabaena limnetica* NIVA-CYA

20 276/6, and c3; *Chlorobium*. The signal intensities for
each spot was determined. The phylogenetic position of
the different strains and probes are visualised by a
phylogenetic tree. The tree was built with the
neighbour-joining algorithm, using Kimura distances.

25 Each branch in the tree has a bootstrap support above
65%.

           Figure 6 - shows a water profile analysis for 7
Norwegian lakes. The lakes range from those with a
relatively low content of biomass (mesotroph) to lakes

30 with high biomass content (eutroph). The signal
intensities relative to the universal probe pUN were
multiplied with a factor obtained from pure cultures to
correct for differences in probe-labelling efficiencies
to obtain the relative abundance of the different

35 genotypes in the samples.

EXAMPLES

Example 1

5   Organisms and sample preparation

       The organisms used are from the Norwegian Institute
for Water Research. The cultivation was performed in
medium Z8. The illumination was provided by fluorescent
lamps exposing the strains with 30 $\mu Em^2 s^{-1}$. Two different
10   Microcystis aeruginosa strains (NIVA-CYA 228/1 and 43)
were used as templates in the development of the assay.
The system was also tested on experimentally modified
water samples collected from Lake Akersvatnet, Norway.
The cells were counted by microscopy in a Fuchst-
15   Rosenthal counting chamber (Carl Hecht, Sondheim,
Germany).

       DNA was either purified with a standard phenol/
chloroform protocol from cell pellets of unialgal
cultures, or by a solid-phase cell concentration and DNA
20   purification protocol (Rudi, K., Larsen F. and Jakobsen,
K.J. in Applied and Environmental Microbiology, (1998),
64, Vol. 1, pp 34-37). In the solid-phase protocol,
cells of cyanobacteria from 1 ml aqueous solution were
adsorbed for 20 minutes onto paramagnetic beads (final
25   volume 2 ml) in a buffer containing 50% isopropanol,
0.75 M ammonium acetate and 1U (the beads in 200 μl
lysis buffer) Dynabeads DNA DIRECT (Dynal A/S, Oslo,
Norway). The magnetic beads and the adsorbed bacteria
were attracted to the side of a 2 ml microcentrifuge
30   tube by a MPC-Q magnet (Dynal A/S). Then 20 μl of 4M
guanidine thiocyanate-1% Sarkosyl was added, and the
incubation continued at 65°C for 10 minutes. The DNA
was precipitated onto the beads by addition of 40 μl 96%
ethanol, with subsequent incubation at room temperature
35   for 5 minutes. Finally, the DNA and bead complex was
washed twice with 500 μl 70% ethanol using the magnet
between each washing. To remove residual ethanol the

complex was dried at 65°C for 5 minutes. The complete
bead and DNA complex was then used in the amplification
reactions.

Competitive PCR (schematically shown in Fig. 1A)

    For selective amplification of genomic DNA from
Microcystis we used the 16S rDNA primers 5'-AGCCAAGTCTG
CCGTCAAATCA-3' (CH) and 5'-ACCGCTACACTGGGAATTCCTG-3'(CI)
(Rudi, K. et al. in Appl. Environ. Microbiol. 63, 2593-
2599). The competitor 5'AGCCAAGTCTGCCGTCAAATCAAGCTG
CCTCACTGCGGAGCTCGGACCAGGAATTCCCAGTGTAGCGGT-3' is an
oligonucleotide with sequences complementary to the PCR
primers CH-CI, and the primer DK (see below) used in the
cyclic labelling reaction. Amplification reactions
using the GeneAmp 2400 PCR thermocycler (Perkin Elmer,
Norwalk, Conn.) contained 10 pmol primers, 6×10^{-9} pmol
competitor, 200 μM of each deoxynucleotide triphosphate,
10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1%
Triton X-100, 1U of DynaZyme DNA polymerase (Finnzymes
Oy, Espoo, Finland) and purified DNA in a final volume
of 50 μl. Prior to amplification, the DNA was denatured
for 4 minutes at 94°C and after amplification an
extension step for 7 minutes at 72°C was included. The
cycling was done for 40 cycles using the parameters;
94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30
seconds.

Cyclic labelling (schematically shown in Fig. 1B)

    Five μl of the PCR products from the competitive
reaction were used in the cyclic labelling reaction.
The deoxynucleotide triphosphates were dephosphorylated
by addition of 100 nm Tris-HCl (pH 8.0), 50 nm $MgCl_2$ and
1U shrimp alkaline phosphatase (USB, Cleveland, OH),
with subsequent incubation at 37°C for 1 hour. Finally,
the phosphatase was inactivated by heating at 96°C for
10 minutes.

    The cyclic labelling reactions were carried out in

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20 μl volumes containing; 3 pmol primer
5'-GTCCGAGCTCCGCAGTGAGGCAG-3' (DK) complementary to the
competitor. 3 pmol primer 5'-
TCTGCCAGTTTCCACCGCCTTTAGGT-3' (DB) complementary to the
Microcystis amplicon, 10 pmol ddATP, *10* pmol ddGTP, 10
pmol ddTTP (Boehringer GmbH, Mannheim, Germany), 7 pmol
fluorescein-12-ddCTP (NEN, Boston, Mass.), 1.25 μl
Thermo Sequenase reaction buffer, 1.1 μl enzyme dilution
buffer, 0.15 μl Thermo Sequenase (Amersham International
plc, Buckinghamshire, England) and 6 μl phosphatase-
treated PCR product. The labelling was done for 25
cycles using the parameters; 95°C for 30 seconds and 50°C
for 4 minutes.

<u>Hybridization and chromogenic detection (schematically
shown in Fig. 1C and D)</u>

One μl (100 pmol/μl) of primer
5'-ACCTAAAGGCGGTGGAAACTGGCAGA-3' (DA) and
5'-CTGCCTCACTGCGGAGCTCGGAC-3' (DJ) were spotted onto
membrane strips (0.4 x 2 cm) GeneScreen (NEN), and then
U.V. cross-linked with 5000 joule/cm². Primer DA is
complementary to primer DB, and primer DJ is
complementary to primer DK. The strips were
prehybridized for 2 hours at 37°C in a prehybridization
solution containing 0.7 x SSC, 1 x SPEP, 5 x Denhardts
and 100 μg/ml heterologous DNA. The products from the
cyclic labelling reactions were added to 0.5 ml
hybridization solution (0.7 x SSC, 1 x SPEP, 1 x
Denhardts, 10% Dextran sulfate and 100 μg/ml
heterologous DNA) in a 2 ml microcentrifuge tube, and
denatured at 95°C for 5 minutes. The strips were added,
and the incubation continued with gentle inversion for 2
hours at 37°C. The membrane strips were washed in 50 ml
(1 x SSC and 1% SDS), then in 50 ml (0.1 X SSC and 0.1%
SDS), and finally twice in 50 ml (0.10 M Tris-HCl [pH
7.5] and 0.15 M NaCl). Each washing was performed by
brief vortexing at room temperature.

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For antibody detection the membrane strips were blocked with 20 ml (0.10 M Tris-HCl [pH 7.5], 0.15 M NaCl and 0.5 % skimmed milk) for 1 hour and incubated in 10 ml of the same buffer containing 1/1000 of Antifluorescein-HRP Conjugate (NEN) for an additional 1 hour. The membrane strips were washed 3 times by brief vortexing in 50 ml (0.10 M Tris-HCl [pH 7.5] and 0.15 M NaCl). The chromogenic reaction was done with the RENAISSANCE 4CN Plus For Chromogenic Detection of HRP for 5 minutes, according to the manufacturers recommendations (NEN).

The relative signal strengths were measured using a CCD video camera (Cohu High Performance CCD Camera, Japan), and analyzed using the Gel-Pro ANALYZER software (Media Cybernetics, Silver Spring, Md.).

Detection assay on defined samples containing purified DNA. By titration experiments the optimum amount of competitor (both for obtaining low detection limits, and for reproducible amplifications) was found to be 6×10^{-9} pmol (i.e. 3600 molecules) per sample test. Accordingly, 6×10^{-9} pmol competitor was used in the testing of the assay on purified DNA from *Microcystis aeruginosa* NIVA-CYA 43. Dilution series of *Microcystis* DNA from approximately 10^7 to 10^0 genomic copies (assuming a genome size of 5 ± 3 Mb (9b)) were used in both the competitive PCR assay (Fig. 2A), and in the subsequent labelling assay (Fig. 2B).

Measurements of the ratio between target relative to competitor products, as determined by agarose gel electrophoresis, gave a quantitative range from 10^5 to 10^2 genomic copies (Fig. 2C). In contrast, the labelling assay the subject of the present invention gave a quantitative range of from more than 10^7 down to 10^2 genomic copies. This is approximately a 100-fold increase in dynamic range compared to agarose gel electrophoresis detection assays (Fig. 2C). As few as

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10 copies could be detected for the labelling assay by
increasing the incubation time of the chromogenic
detection reaction from 5 to 30 minutes. Live video
capture of the chromogenic detection reaction may be
used to give a quantitative range down to this level.
That is, time-curves from 0 to 30 minutes for the colour
density of the target and competitor spots can be used
to extrapolate the relative signal intensities, although
the competitor spot is colour-density saturated after 30
minutes.

**Effect on the quantitative range by the number of
cycles in the labelling reaction.** The cyclic labelling
reaction increased the quantitative range of the assay
compared to direct detection of the amplified DNA. For
competitive PCR - using a logarithmic scale for the
target concentration - the ratio of competitor and
target signals resulted in a sigmoid curve with
relatively narrow quantitative range (see Fig. 2C). A
sigmoid curve was also obtained by performing the cyclic
labelling assay with just a few labelling cycles.
However, by increasing the number of labelling cycles,
the competitor or the target oligonucleotides were label
saturated (all the probes were labelled) at each of the
dilution series endpoints, respectively, leading to
curve with a wider quantitative range (see Fig. 2C).
Increasing the cycle number further resulted in a curve
which was flatter in the middle because of label
saturation of both oligonucleotides at this location.

Quantification of *Microcystis* in water samples.
The complete quantitative assay which includes the
solid-phase cell concentration and DNA purification
method was carried out on dilution series (10^5-10^0
cells/ml) of *Microcystis aeruginosa* (strains NIVA-CYA 43
and 228/1). *Planktothrix agardhii* NIVA-CYA 29
(filamentous) and *Anabaena lemmermanii* NIVA-CYA 83/1

(filamentous and heterocyst containing) were used as controls of the reaction specificity. *Microcystis* cultures were diluted in pure water, water containing 10^5 cells/ml of *Planktothrix agardhii* NIVA-CYA 29, water containing 10^5 cells/ml of *Anabaena lemmermanii* NIVA-CYA 83/1, and water sampled from Lake Akersvatnet, Norway.

There were no significant differences in either specificity or sensitivity of the assay for the different conditions tested. With 6 x 10^{-9} pmol competitor we obtained a quantitative range spanning from more than 10^5 down to 10^2 cells/ml in all cases (results for *Microcystis aeruginosa* NIVA-CYA 43 are shown in Fig. 3). By lengthening the incubation time for the chromogenic detection reaction from 5 to 30 minutes we could detect down to 10 cells/ml.

The detection curve for the complete detection assay (including solid-phase cell concentration and DNA purification) has about the same slope as the curve obtained for the dilution series of purified DNA (compare Figs. 2C and 3), indicating that the cell concentration and DNA purification (sample preparation) method is not affected by the sample composition. Furthermore, these results also show that the solid-phase cell concentration and DNA purification method can be used for quantitative analysis directly from water-samples.

The detection limit was dependent on the amount of competitor used, with a lower limit (6 x 10^{-9} pmol) competitor. Increasing the amount of competitor 10-fold (6 x 10^{-8} pmol) also resulted in about 10-fold increased detection limit (Fig 4). However, lowering the amount to 6 x 10^{-10} pmol gave irreproducible results. Thus, we conclude that 6 x 10^{-9} pmol was also the optimum amount of competitor both for obtaining low detection limits and reproducible detections for the complete assay on water-samples.

Complete assay for quantification of cyanobacteria
in water. Cyanobacteria belonging to the genus
Microcystis can produce several different types of
toxins, with the hepatotoxic microcystins as the most
potent. This toxin not only causes an acute poisoning
by liver damage, but can also be a carcinogenic tumor
promoter on long term exposure to low doses. Thus, a
continuous monitoring system to screen for the presence
of the organisms producing this toxin is important.

Health authorities in Australia (New South Wales
Blue-green Algae Task Force, 1992) have already adopted
a three-level alert system based on cyanobacterial cell
counts in water. Level 1 is 500-2000 cells/ml, at this
level water authorities are alerted and water sampling
for monitoring is increased. Level 2 is 2000-15000
cells/ ml, at this point toxicity testing is carried
out. Level 3 is above 15.000 cells/ml, if activated
carbon is not available then the water may be declared
unsafe for human consumption. With a detection limit
for *Microcystis* of 100 cells/ml and a quantitative range
of more than three orders of magnitude, the methods of
the invention described herein are suited for the
monitoring of both low *Microcystis* concentrations and
the detection of potential toxic waterblooms in drinking
water.

Development of multiplex assays. The general
sample preparation with the solid-phase cell
concentration and DNA purification method, combined with
the specificity in the detection method, enable
multiplex determinations. Using competitive PCR,
multiple targets may be quantified by using e.g.
universal 16S rDNA primers in the competitive reaction.
Then, different oligonucleotide probes can be labelled
based on signature sequences for distinct bacterial
groups, and finally, the ratios of the competitor signal
can be compared to the signals for each of the bacterial

groups.

Example 2

Unlike traditional classification based on
morphology/cytology, classification based on nucleic
acid sequences reflect the evolution and phylogeny of
the organisms. 16S rDNA-targeted probes were
constructed to detect the major cyanobacterial groups
Microcystis, *Planktothrix*, *Anabaena* and *Aphanizomenon*.
Probes were also constructed to differentiate groups of
different phylogenetic depth. A probe was constructed
to detect the *Nostoc* group (which includes *Anabaena* and
Aphanizomenon), and one probe was constructed to detect
all eubacteria (including chloroplasts). The
specificity of the constructed probes was tested on
unialgal cultures. This information was subsequently
used to determine the relative distribution and
abundance of the targeted organisms in 7 lake
communites.

Organism and sample preparation.

The following unialgal cultures from the Norwegian
Institute for Water Research was used; *Aphanizomenon
gracile* NIVA-CYA 103, *Anabaena lemmermannii* NlVA-CYA
266/1, *Nostoc* sp. NIVA-CYA 124, *Phormidium* sp. NIVA-CYA
203, *Planktothrix prolifica* NIVA-CYA 320, *Microrystis
aeruginosa* NIVA-CYA 143, *Microcystis flos-aquae* NIVA-CYA
144, *Pseudanabaena limnetica* NIVA-CYA 276/6. The
cultivation was performed in medium Z8 [Norwegian-
institute-for-water-research, Oslo (1990) Culture
Collection of algae, catalogue of strains]. The
illumination was provided by fluorescent lamps exposing
the strains with 30 $\mu Em^{-2}s^{-1}$. Aliquots of dense cultures
(1 ml containining approximately 10^7 cells/ml) were
pelleted in a microcentrifuge at 5,000 rpm for 10 min
and immediately frozen at $-80^\circ C$. The DNA was purified

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from the frozen pellets with the magnetic bead-based DNA
Direct DNA isolation Kit (Dynal A/S, Oslo, Norway),
using a protocol modified for the purification from
cyanobacteria [Rudi K., Kroken, M., Dahlberg, O.J.,
Deggerdal, A., Jakobsen, K.S. and Larsen, F. (1997)
Biotechniques **22**, 506-11].

The samples collected from the field were
immediately conserved in isopropanol, as described for
the cell concentration step below. The samples were
then transported to the laboratory, and processed
further. The DNA was purified by a solid-phase cell
concentration and DNA purification protocol previously
developed by Rudi et at. (1998). In the solid-phase
protocol, cells from 0.8 ml aqueous solution were
adsorbed for 20 min onto paramagnetic beads (final
volume 2 ml) in a buffer containing 50% isopropanol,
0.75 M ammonium aeetate and 1U (the beads in 200 μl
lysis buffer) Dynabeads DNA DIRECT (Dynal A/S, Oslo,
Norway). The magnetic beads and the adsorbed bacteria
were attracted to the side of a 2 ml microcentrifuge
tube by a MPC-Q magnet (Dynal A/S). Then 20 μl 4M
guanidine thiocyanate-1% Sarkosyl was added, and the
incubation continued at 65°C for 10 min. The DNA was
precipitated onto the beads by addition of 40 μl 96%
ethanol, with subsequent incubation at room-temperature
for 5 min. Finally, the DNA and bead complex was washed
twice with 500 μl 70% ethanol using the magnet between
each washing. To remove residual ethanol the complex
was dried at 65°C for 5 min. The complete bead and DNA
complex was then used in the amplification reactions.

PCR amplification.

Ribosomal DNA was amplified, using the universal
primer set CC - CD. Amplification reactions using the
GeneAmp 2400 PCR thermocycler (Perkin Elmer, Norway,
Conn.) contained 10 pmol primers, 200 μM of each
deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.8),

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1.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton X-100, 1U of
DynaZyme DNA polymerase (Finnzymes Oy, Espoo, Finland)
and purified DNA in a final volume of 50 µl. Prior to
amplification, the DNA was denatured for 4 min at 94°C
and after amplification an extension step for 7 min at
72°C was included. The cycling was done for 35 cycles
using the parameters; 96°C for 15 s, 70°C for 30 s and
72°C for 1 min.

Cyclic labelling.

        Twenty µl of the PCR products from the
amplification reactions were used in the cyclic labeling
reaction. The deoxynucleotide triphosphates were
dephosphorylated by addition of 100 nm Tris-HCl (pH
8.0), 50 nm MgCl$_2$ and 1 U shrimp alkaline phosphatase
(USB, Cleveland, OH), with subsequent incubation at 37°C
for 1 hour. Finally, the phosphatase was inactivated by
heating at 96°C for 10 min.

        The cyclic labeling reactions were carried out in
80 µl volumes containing 3 pmol of each of the primers
in Table 1 below, 10 pmol ddATP, 10 pmol ddGTP, 10 pmol
ddTTP (Boehringer GmbH, Mannheim, Germany), 7 pmol
fluorescein-12-ddCTP (NEN, Boston, Mass.), 1.25 µl
Thermo Sequenase reaction buffer, 1.1 µl enzyme dilution
buffer, 0.15 µl 32 U/µl Thermo Sequenase (Amersham
Pharmacia plc, Buckinghamshire, England) and 25 µl
phosphatase-treated PCR product. The labeling was done
for 10 cycles using the parameters; 95°C for 30 sec and
50°C for 4 min.

Table 1 - Oligonucleotide probes

Probes	probe sequences[1]
pKO	5'CCTCTGGTACCGTCAGGTTGCTTTCACAA3'
pMI3	5'CCCTGAGTGTCAGATACAGCCCAGTAG3'

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pMI2	5'GCAGGTGGTCAGCCAAGTCTGC3'
pDK	5'TCTGCCAGTTTCCACCGCCTTTAGGT3'
pPL1	5'TACAGGCCACACCTAGTTTCCATCGTTTAC3'
pAL	5'CTGCTGTTAAAGAGTCTGGCTCAACCAGAT3'
pAP	5'CCCCTAGCTTTCGTCCCTCAGTGTCAGT3'
pNOS	5'GCTCAACCARATMARAGCAGTGGAAACTA3'
pPL2	5'CAATCATTCCGGATAACGCTTGCATCC3'
pUN	5'CCGTMTTACCGCGGCTGCTGGCA3'

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[1]the primers complememtary to these probe sequences were spoted on the membranes

Hybridization and chromogenic detection.

Half a μl (100 pmol/μl) of primers complementary to the primers used in the labeling reaction were spotted onto membrane strips (4 x 5 cm) Hybon (Amersham), and then U.V. cross-linked with 5000 joule/cm^2. The strips were prehybridized for 2 hours at 37°C in a prehybridization solution containing 0.7 x SSC, 1 x SPEP, 5 x Denhardts and 100 μg/ml heterologous DNA. The products from the cyclic labeling reactions were added to 0.5 ml hybridization solution (0.7 x SSC, 1 x SPEP, 1 x Denhardts, 10% Dextran sulfate and 100 μg/ml heterologous DNA) in a 2 ml microcentrifuge tube, and denatured at 95°C for 5 min. The strips were added, and the incubation continued with gentle inversion for 2 hours at 37°C. The membrane strips were washed in 50 ml (1 x SSC and 1% SDS), then in 50 ml (0.1 x SSC and 0.1% SDS), and finally twice in 50 ml (0.10 M Tris-HCl [pH 7.5] and 0.15 M NaCl). Each washing was performed by brief vortexing at room temperature.

For antibody detection the membrane strips were blocked with 20 ml (0.10 M Tris-HCl [pH 7.5], 0.15 M NaCl and 0.5% skimmed milk) for 1 hour and incubated in 20 ml of the same buffer containing 1/1000 of Antifluorescein-HRP Conjugate (NEN) for an additional 1 hour. The membrane strips were washed 3 times by brief

vortexing in 50 ml (0.10 M Tris-HCl [pH 7.5] and 0.15 M
NaCl). The chromogenic reaction was done with the
RENAISSANCE 4CN Plus For Chromogenic Detection of HRP
for 5 min, according to the manufacturers
5   recommendations (NEN).

The relative signal strengths were determined by
scanning the membranes with an Agfa snapscanner 600, and
analyzed using the Gel-Pro ANALYZER sofware (Media
Cybernetics, Silver Spring, Md.).

10

Validation of the probe labeling assay on pure cultures.

The probe labeling assay was applied on 8 different
cyanobacterial strains representing the major lineages.
The outgroup *Chlorobium* sp. was included as an
15   additional control of the specificity of the probes.
Approximately 5% of the DNA purified form the unialgal
cultures were amplified with the universal primer-pair
CC-CD in a 50 μl PCR reaction volume. The products were
visualized on a 1.5% ethidium bromide-stained agarose
20   gel, prior to the labeling, to confirm the amplification
reaction. All of the samples were uniformly amplified,
yielding a strong band at approx. 600 bp, with no
visible additional bands.

Related to the probe-labeling assay, the universal
25   probe pUN was labeled relatively uniformly (with an
exception for *Pseudanabaena limnetica* NIVA-CYA 276/6)
for the strains tested (Fig. 5A). This probe was also
labeled for the outgroup *Chlorobium* sp.. Except for the
probe pPL2, the different cyanobacteria specific probes
30   gave signals as expected from their phylogenetic
position (Fig. 5B and C). The low specificity of pPL2
may be due to the fact that the hybridization
temperature used was not optimal for this probe.
Generally, however, there was a high signal to noise
35   ratio in the assay. For instance, there is only one
base-pair difference between *M. aeruginosa* strains NIVA-
CYA 143 and 144. Probe pMI2 distinguished NIVA-CYA 143

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and 144 with a signal to noise ratio of 80.

5   The incorporation efficiency of fluorescently labeled dideoxy nucleotides is sequence dependent. There are also differences in the probe-labeling due to probe-target hybridrzation efficiencies. The base composition and the melting point, in addition to the sequences flanking the probe region, affect the probe

10   hybridisation. The different labeling efficiencies ranged from 1.0 to 5, as determined relative to the labeling of the universal probe pUN for the respective probes developed in this work. For the respective probes, the efficiencies were as follows; pKO - 1.7, pM13 - 2.2,

15   pM12 - 1.4, pDK - 1.9, pPL1 - 4.0, pAL - 1.6, pAP -3.3, pNOS - 4.3, and pPL2 - 4.9.

<u>Distribution of cyanobacteria from 7 selected localities.</u>

The generality of the sample preparation and probe

20   labeling strategies were tested on water samples from 7 different localities, ranging from water with a moderate level of plant biomass (mesotrophic) to water with a high biomass content (eutrophic).

Three samples were collected from each sampling

25   site. The cell concentration and DNA purification were performed as described above for all three samples in parallel. Ribosomal DNA was amplified from 90,9, and 1% of the purified material. Amplification reactions were obtained for Østensjøvatnet v Oslo, Gjærsjøen and

30   Årungen for 90% of the purified DNA. For 9% of the material all the samples were amplified, except the samples from Langen, while for 1% amplification reactions were obtained for all the samples.

The average signal strength and standard deviation

35   for the probe labelling were calculated for each investigated locality (based on the three collected samples). Since the different probes are not labelled with equal efficiently, this was adjusted for based on

the relative labelling efficiencies obtained from the
analyses of pure cultures (Fig. 6). The bars in this
figure show the approximate relative composition of the
determined genotypes in the sample. Because there is an
unknown component of heterotrophic bacteria in some of
the cultures used for correlation, the quantitation of
the respective organisms may be an overestimate.

As a further verification of the probe-labelling
assay the samples were also examined by microscopy.
Here, the dominant species for each locality was
determined based on morphological and cytological
characteristics, according to the criteria given by
Skulberg et al [Skulberg, O.M. Carmichael, W.W., Codd,
G.A. and Skulberg R. (1993) Algal toxins in seafood and
drinking water pp 145-164 Ed. Falconer I.R., London
Academic Press Ltd]. There was generaly a good
correlation between the groups of organisms in the
sample, as determined by the genetic and the microscopic
analyses. The 16S rDNA gene used in the genetic
analysis, however, does not have enough sequence
variation for discrimination to the species level. In
addition, we could not distinguish between the
morphologicaliy defined genera Anabaena and
Aphanizomenon with the probe-labelling assay. The
reason for this is that the two positions used for
discrimination do not follow the species designation.
This can be due to that there have been several
independent substitutions for the respective positions,
that there have been recombination events in the 16S
rDNA between the two probes, or that the species
definition is not phyletically relevant. The
hierarchical probe pNOS, on the other hand, seemed
specific for the Nostoc group - including Anabaena and
Aphanizomenon.

The Planktothrix specific probe pPL1 gave a
relatively strong signal with the probe-labelling assay
for lake Gjersøen, while this organism was not detected

by the microscopic examinations of water from this lake.
This diiference may be explained by the fact that
samples for microscope analysis was concentrated through
a 2 μm plankton net before the analysis, while for the
genetic analysis the sample was analyzed directly.
Furthermore, the cyanobaceria may not be distributed
evenly in the lake, which may also explain the
diiferences in the probe labeling and the microscopic
examinations.